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Growth

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14. ABSTRACT The Pim protein kinase is over expressed in prostate cancers. To clarify the role of this protein in regulating prostate cancer growth we have investigated its mechanism of action. Additionally, we have studied the ability of small molecule inhibitors of Pim developed in our laboratory to block the growth stimulatory affects of this protein kinase. We find that Pim-1 is able to decrease the levels of the cell cycle inhibitory protein p27 by phosphorylating Skp-2, the protein that regulates the level of p27. Phosphorylation of Skp-2 prolongs the half-life of Skp-2, increases its levels, and enhances its ability to degrade p27. Pim also functions to stimulate the phosphorylation of the Cdc27 protein also inhibiting its function. Since active Cdc27 is needed for the destruction of Skp-2, this also increases Skp-2 and decreases the levels of p27. Low levels of p27 allow prostate cancer cells to rapidly transit the cell cycle. We find that our chemical inhibitor of Pim, SMI-4a, is able to reverse the activity of Pim-1 and increase the levels of p27. This data suggests that inhibition of Pim-1 by these agents would function to block prostate cancer growth by inhibiting transit through the cell cycle. Thus, our agent has potential activity to treat this cancer.					
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INTRODUCTION (subject, purpose, and scope)

We hypothesize that overexpression of the Pim protein kinases drives down the levels of the p27Kip1 (p27) protein and enhances the growth to prostate tumors. P27 normally functions to inhibit the growth of tumor cells by blocking the activity of the cyclin dependent protein kinases that drive cell growth. We have previously demonstrated using animal and tissue culture models that the overexpression of the Pim protein kinase even in transformed human prostate tumors will stimulate the growth of these cells. In addition, others have demonstrated that using Kaplan-Meier analysis that patients with a higher level of Pim are more prone to metastasis. We suggest that the c-Myc protooncogene works in concert with low levels of p27 to drive tumor growth. Further, we hypothesize that small molecule inhibitors of Pim protein kinase have the potential to reverse these effects. As a research team involving chemists and biologists, we have developed novel benzylidene thiazolidine-2,4-diones that inhibit Pim kinase activity and demonstrated that these compounds are capable of blocking Pim kinase activity both in tissue culture and in animals. The goal of these experiments is three fold. Our first goal is understand the mechanism by which Pim protein kinases regulate the cell cycle and to demonstrate that our specific inhibitors can reverse this growth stimulatory activity. The second goal of this work is to determine whether in human prostate cancers changes in the cell cycle regulators correlates with the level of Pim protein kinases. Third, to evaluate whether maximal induction of p27 and inhibition of tumor growth requires small molecule inhibition of both Pim-1 and Pim-2 protein kinases.

BODY

Our research efforts have focused on specific portions of the Statement of Work, and in an attempt to bring together data that will satisfy the needs of a peer reviewed publication. For this reason we have focused on Task 2(a, b) in year 1 and Task 1 and 2 (a, b) in year 2. Although these tasks were not evaluated in the order of the prescribed Statement of Work, this analysis does form a coherent explanation for the activity of the Pim protein kinase. The results of these experiments are documented in the enclosed paper and have been submitted to the Journal of Biologic Chemistry. Rather than separating these tasks into individual goals for simplicity we have combined this description.

Task 2(a, b) in year 1 and Task 1 and 2 (a, b) in year 2- We have found that the overexpression of active Pim1 protein kinase but not kinase dead decreases the level of p27 protein. To evaluate the mechanism we have focused our attention on the Skp2 protein which bind directly to p27 and activates the E3 ligase which ubiquitinates p27 leading to its degradation. We demonstrate that knocking down Pim-1 kinase increases Skp-2 and decreases p27 levels (*Fig. 1*) (NOTE: All Figures begin at Page 33). Similarly, we find that inhibition of Pim kinases with increasing doses of our small molecule Pim kinase inhibitor, SMI-4a [called in the past D5], decreases the level of Skp-2 and increases p27 protein (*Fig. 2*). In tissue culture, overexpression of Pim-1 in normal mouse prostate epithelial cells increased Skp-2 levels while decreasing p27 protein (*Fig. 3*). Experiments carried out in which the half-life of the Skp-2 protein was evaluated by adding cycloheximide to cells demonstrated that the expression of Pim-1 markedly increased the

half-life of Skp-2 protein (Fig. 4). These results suggest that the regulation of Skp-2 by Pim-1 decreases p27 levels. This effect is inhibited by SMI-4a, the Pim kinase inhibitors we have developed.

To evaluate how Pim and Skp-2 may interact we examined whether these proteins could coimmunoprecipitate. As seen in *Fig. 5*, both kinase active and dead Pims will bind directly to the Skp-2 protein. Immunoprecipitating Pim-1 from serum stimulated cells demonstrates that Pim and Skp-2 associate in a cell cycle dependent manner (*Fig. 6*). To evaluate how Pim-1 might effect the half-life of the Skp-2 protein we evaluated whether Pim-1 could regulate the ubiquitination of Skp-2. We find that if a tagged Skp-2 and 6xHis-ubiquitin plasmids are transfected into cells along with Pim-1 that the ubiquitination of Skp-2 is inhibited, explaining the increased levels of this protein (*Fig. 7*). In addition, we find that Pim-1 can phosphorylate Skp-2 and the addition of our inhibitor, SMI-4a, can block the ability of Pim-1 to modify the Skp-2 protein (*Fig. 8*). Together these results suggest that the Pim protein kinase binds to Skp-2 and inhibits its ubiquitination possibly in part secondary to the ability of Pim-1 to phosphorylate Skp-2.

We have used GST fusion proteins to evaluate the phosphorylation of Skp2. Skp-2 is thought to be phosphorylated on serine 64 and 72. We have defined a new phosphorylation site on this protein that is modified by Pim-1. Shown in *Figure 9* is the conservation of threonine 417 across species. If this site is mutated in Skp-2 it partially decreases the phosphorylation of this protein (*Fig. 10*). This result suggests that Skp-2 is a target of Pim and that 417 and potentially other sites are phosphorylated. Expanding this experimental approach mutation of S64 or S72 also inhibits Pim phosphorylation of Skp-2 (*Fig. 11*). Our small molecule Pim inhibitor, SMI-4a, blocks the Pim-induced phosphorylation of this protein (*Fig. 12*). To evaluate the potential effects of these phosphorylations on the half-life of this protein each of these three sites was mutated, transfected into cells, which were then treated with cycloheximide and the half-life of the protein examined by Western blotting (*Fig 13a,b*). We find that each of these phosphorylation mutants shortens the half-life of these proteins (*Fig. 13 a,b*). Another approach to examining the importance of these phosphorylation sites is to cotransfect Skp-2 and p27. If Skp-2 is active then p27 levels will be lowered. We find in *Figure 13c* that mutation of T417 and to a greater extent S64 but not S72 affects the level of p27. In conclusion, Pim phosphorylates Skp-2 on S64, S72, and T417 and this phosphorylation regulates the half-life of Skp-2 and thus the levels of p27. Our Pim inhibitors block this phosphorylation and thus would raise the levels of p27.

To discern how Pim might regulate Skp-2 ubiquitination, we examined in detail the APC/C complex. either Cdc20 or Cdc27. This E3 ligase is regulated by. We find that the addition of Pim-1 to cells dissociates the Cdc27 regulator protein from the APC/C complex (*Fig. 14a*), and knocking Pim-1 down using siRNA increases the amount of this protein (*Fig. 14b*). We find that Pim-1 is capable of phosphorylating Cdc27 but not Cdc20 both *in vitro* and when over expressed in cells (*Fig. 15a, b*).

Based on the interaction between and Pim-1 protein kinase and Skp-2 we have transfected either one or both of these proteins into prostate cells and examined the levels of p27 and

the location of cells in the cell cycle. We find that cells expressing both protein have the lowest levels of p27, the fewest cells in G1 and the most cells in the G2 phase of the cell cycle (*Fig. 16a*). This result is highly significant (*Fig. 16b*). Our small molecule inhibitor of Pim-1 (SMI-4a) can block the ability of Skp-2 or serum to drive cells into the G2 phase of the cell cycle (*Fig. 16c*) and stimulate an increase in p27.

We include a model for how the Pim kinase can regulate the levels of Skp-2 (*Fig. 17*). Pim-1 can phosphorylate both Skp-2 and CDC27 and lead to their destruction. This combined effect can be inhibited by SMI-4a. Our novel Pim inhibitor can be developed as a compound that increases the levels of p27, blocks progression through the cell cycle, and inhibits tumor growth.

KEY RESEARCH ACCOMPLISHMENTS

- **Increases in Pim-1 kinase decreases Skp-2 levels and thus allows p27 protein to increase.**
- **The changes in these proteins are inhibited by the small molecule Pim-1 inhibitor we have developed.**
- **Pim-1 binds to Skp-2 and inhibits its ubiquitination and thus degradation.**
- **Skp-2 is phosphorylated by Pim-1 and our small molecule Pim inhibitors block this effect.**
- **Threonine 417 in Skp-2 is identified as a new phosphorylation site in Skp-2**
- **Serines 64 and 72 are mapped as Pim phosphorylation sites.**
- **Pim-directed phosphorylation controls the half-life and activity of Skp-2.**
- **Pim regulates the phosphorylation of Cdc27 to bind to the APC/C, and controls the phosphorylation of this protein.**
- **Pim-1 plus Skp-2 enhances transition through the cell cycle.**
- **Pim kinase inhibitory compounds block the ability of cells to transverse the cell cycle.**

REPORTABLE OUTCOMES

Abstract

Cen B, Zemskova M, Beharry Z, Smith CD, and Kraft AS. Potent protein kinase inhibitors block Pim kinase mediated increase in prostate epithelial cell migration, regulation of p27 protein half-life and secretion of hepatocyte growth factor. Abstr., Proc. Am. Assoc. Cancer Res., 160:A249, 2009.

Paper

Cen B, Sandeep M, Zemskova M, Beharry Z, Lin YW, Cramer SD, Lilly M, and Kraft AS. Regulation of SKP2 Levels by the PIM-1 Protein Kinase. Submitted to J Biol Chem 2010. Under review.

CONCLUSIONS

We can conclude from this research that Pim remains an important target for treatment of human prostate cancer. The Pim-1 protein kinase clearly plays a role in controlling the levels of p27 and transition through the cell cycle. *This work demonstrates that small molecule inhibitors of this protein kinase, such as SMI-4a, will have activity as a treatment for prostate cancer.*

Based on these results the experiments to be carried out in the next year come into focus. In terms of the Statement of Work we will need to carry out Year 1 task 1, 2c, and 3. Although these tasks are out of order they come into heightened importance and focus based on the results presented here. For example in Task 1 it is important to evaluate in human samples whether the levels of Pim-1 do actually correlate with increased Skp-2 and low levels of p27. One approach to this task is to use immunoperoxidase staining of human tissues. Another approach that may yield reasonable data is the isolation of mRNA from human tissues by laser capture microscopy and the evaluation of the levels of these mRNAs by qT-PCR. It will also be important to carry out task 3 and test these inhibitors in animal models of human prostate cancer and assess whether SMI-4a at maximal doses is sufficient to inhibit tumor growth based on cell cycle blockade

Human prostate cancer cell lines and tumors have all three Pims, 1, 2 and 3. Further research needs to qualify whether inhibition of one or all three Pims is needed to inhibit cell cycle movement to a greater extent than inhibition of Pim-1 alone. Answering this question will greatly assist in the development of targeted therapies to inhibit prostate cancer growth.

APPENDIX A

REGULATION OF SKP2 LEVELS BY THE PIM-1 PROTEIN KINASE

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Running head: Pim-1 regulates Skp2 levels

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The Pim-1 protein kinase plays an important role in regulating both cell growth and survival, and enhancing transformation by multiple oncogenes. The ability of Pim-1 to regulate cell growth is mediated, in part, by the capacity of this protein kinase to control the levels of the p27, a protein that is a critical regulator of cyclin-dependent kinases that mediate cell cycle progression. To understand how Pim-1 is capable of regulating p27 protein levels, we focused our attention on the SCF^{Skp2} ubiquitin ligase complex that controls the rate of degradation of this protein. We find that expression of Pim-1 increases the level of Skp2 through direct binding and phosphorylation of multiple sites on this protein. Along with known Skp2 phosphorylation sites including Ser64 and Ser72, we have identified Thr417 as a unique Pim-1 phosphorylation target. Phosphorylation of Thr417 controls the stability of Skp2 and its ability to degrade p27. Additionally we find that Pim-1 regulates the APC/C complex that mediates the ubiquitination of Skp2. Pim-1 phosphorylates Cdh1 and impairs binding of this protein to another APC/C complex member, CDC27. These modifications inhibit Skp2 from degradation. Marked increases in Skp2 caused by these mechanisms lower cellular p27 levels. Consistent with these observations, we show that Pim-1 is able to cooperate with Skp2 to signal S phase entry. Our data reveal a novel Pim-1 kinase-

dependent signaling pathway that plays a crucial role in cell cycle regulation.

The Pim family of serine/threonine kinases regulates the growth and survival of cells, and plays a role in enhancing the transformed phenotype driven by oncogenes including Myc and Akt (1-3). As the Pim kinases are elevated in human tumors, including prostate, leukemia, and pancreatic cancer, and appear to be useful in distinguishing benign from malignant tumors (4), it has been suggested that they play a role in the growth or progression of these malignancies (5-6). In prostate cancer, decreased Pim-1 expression correlated significantly with measures of poor outcome and was found to be associated with a higher cumulative rate of prostate specific antigen (PSA) failure and a strong predictor of PSA recurrence (4). Based on crystal structural analysis (7-11), the Pim family of kinases appears to be constitutively active and not regulated by a kinase cascade. To explain the ability of the Pim protein kinases to regulate growth and survival, research has initially focused on the ability of these protein kinases to regulate CDC25A and CDC25C, p21^{Waf1} and the C-TAK1 protein kinase (12-14). Recently, Pim-1 has been shown to increase the cyclin-dependent kinase-2 activity, by decreasing the levels of p27^{Kip1} (p27) protein (15). Similarly, we have demonstrated that small molecule inhibitors of Pim-1 both translocate the p27 protein to the nucleus, and markedly increase its levels (16-17), suggesting that inhibiting Pim-1

activity may regulate the cell cycle by controlling p27 levels and localization.

The SCF^{Skp2} ubiquitin ligase (Skp1/cullin/F-box protein) targets cell cycle negative regulators p27, p21^{Waf1}, and p130 (18) to the proteasome for degradation and controls progression through the cell cycle. A key protein in this complex Skp2 binds phosphorylated p27 and is responsible for its destruction. The fact that increased Skp2 expression is frequently found in many cancers (19-20) and Skp2 overexpression can drive cell transformation, suggests the importance of the levels of this protein in regulating cell growth (19,21-22). The amount of the Skp2 protein in cells is tightly regulated by multiple pathways, including phosphorylation and proteasome degradation. The anaphase-promoting complex or cyclosome (APC/C) is active from mitosis to late G1 (23-24) and functions as the E3 ligase for this protein when activated by Cdh1 (25-26). Phosphorylation of Skp2 by CDK2 (27) and Akt1 (28-29) on Ser64 and Ser72 protects it from degradation by the APC/C^{Cdh1} complex and elevates the levels of this protein. However, the exact role of Skp2 Ser72 phosphorylation is currently debated (30-31). Further studies are required to fully elucidate the mechanisms by which cells regulate Skp2 levels.

Here we demonstrate that Pim-1 kinase activity stabilizes and increases the levels of Skp2 protein, thus decreasing p27 levels, and promoting cell cycle progression. Pim-1 both binds Skp2 and phosphorylates it on Ser64 and Ser72, but also on a novel site, Thr417. Furthermore, Pim-1 phosphorylates Cdh1, impairing its association with CDC27 and inhibiting APC/C activity, thus protecting Skp2 from degradation.

Experimental Procedures

Antibodies, drugs, and reagents- Anti-Pim-1 (19F7) antibody was produced and purified in this laboratory. Anti-cyclin E (HE12), anti-Met (25H2), anti-Phospho-Met (D26), Myc tag (71D10), anti-AKT, and anti-Phospho-AKT (S473) antibodies were purchased from Cell Signaling Technology. Anti-p27 (C19), anti-CDC27 (AF3.1), and anti-cyclin B1 (H-433) were from Santa Cruz Biotechnology. Anti-β-actin (AC-15), anti-FLAG M2, anti-HA (HA-7), and anti-β-tubulin (TUB 2.1)

antibodies were from Sigma. Anti Skp2 antibody was from Invitrogen/Zymed. Anti-His tag antibody was from Qiagen. Anti-Cdh1(DH01) antibody was from Abcam. Anti-Lamin B antibody was from Calbiochem.

Roscovitine, and reagents for the *in vitro* ubiquitination assay were from Biomol. Cycloheximide, MG132, LY294002, wortmanin, nocodazole and thymidine were from Sigma. GSK690693 was kindly provided by Glaxo Smith Kline.

Recombinant human HGF was from Antigenix America. Active GST-tagged Pim-1 was from SignalChem. Active his-tagged human Pim-1 was purified from *E. coli* using a Calbiochem Ni-NTA column. GST and GST-Skp2 proteins were purified from *E. coli* using GE Healthcare Glutathione Sepharose 4B resin.

Plasmids- A Pim-1 siRNA plasmid and the control plasmid were described previously (32). pGIPZ Pim-1 shRNA constructs were from Open Biosystems.

pCMV-Skp2 plasmid expressing FLAG-tagged Skp2 was kindly provided by Dr. Liang Zhu (33). Site-directed mutants were prepared using PCR based on this plasmid. HA-Cdh1 and HA-Cdc20 plasmids were described elsewhere (34). The Ubc3 and ubiquitin plasmids have been previously described (35). The HA-Pim-1 and FLAG-Pim-1 constructs was generated by subcloning murine Pim-1 cDNA into pcDNA3 vector, and the K67M (HA-tagged) mutant was constructed using PCR. And an N-terminally truncated mutant (NT81) of Pim-1 was described previously (36). Lentiviral expression constructs pLEX-Pim-1 and pLEX-Skp2 were obtained by subcloning human Pim-1 and Skp2 cDNAs into pLEX vector (Open Biosystems). A human Pim-1 construct, pcDNA3-Pim-1 was described elsewhere (32).

Cell Culture, transfections, transductions, and cell synchronization- Cell lines were grown in RPMI (PC3) or DMEM (HeLa, HEK293T, Rat1, and MEFs). The triple knockout mouse of the Pim-1,-2,-3 genes used to isolate MEFs were described previously (17). Mouse prostate epithelial cells were isolated as described (37). HEK293T cells were transfected by the calcium phosphate method

and HeLa cells were transfected with lipofectamine 2000 reagent. Lentiviruses were produced and transduced into Rat1 cells using kits from Open Biosystems.

For synchronization experiments, HeLa cells were treated with 2 mM thymidine for 18 h, washed and released into fresh medium for 9 h. A second thymidine treatment was added to block cells at the G1/S transition. Mitotic HeLa cells were obtained by treating cells with 2 mM thymidine for 24 h, washing and releasing them into fresh media for 3h. The cells were then treated with 100 ng/ml nocodazole for 12 h.

Ubiquitination assays- *In vitro* p27 ubiquitination assays were performed essentially as described (38). In brief, the Skp2-SCF complex was expressed and purified from insect cells (39) and mixed with *in vitro*-translated ³⁵S-p27 that had previously been incubated with cyclin E/Cdk2 along with methylated ubiquitin and ubiquitin aldehyde for 60 min at 30 °C. The reaction was stopped with 2 × SDS sample buffer and run on polyacrylamide gels. *In vivo* ubiquitination assays were performed as described (40). HEK293T cells were transfected with the indicated plasmids for 24 h, treated with 10 μM MG132 for 6 h, and lysed in denaturing buffer (6 M guanidine-HCl, 0.1 M Na₂HPO₄/NaH₂PO₄, 10 mM imidazole). The cell extracts were then incubated with nickel beads for 3 h, washed, and subjected to immunoblot analysis.

***In vitro* and *in vivo* phosphorylation assay-** FLAG-Skp2 or its mutants were immunoprecipitated with anti-FLAG antibody from HEK293T cells. Immune complexes were washed three times in RIPA lysis buffer (150 mM NaCl, 10 mM Tris-HCl pH 7.5, 1% Nonidet P40, 0.5% deoxycholate, 0.1% SDS), then washed twice in 1× kinase buffer (25 mM Tris-HCl pH 7.5, 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na₃VO₄, 10 mM MgCl₂, 2 μM unlabelled ATP), and incubated with 0.5 μg recombinant active Pim-1 kinase and 2 μCi of [γ-³²P]ATP in 3 μl total reaction buffer for 30 min at 30 °C. Phosphorylation of Cdh1 or Cdc20 was detected using *in vitro* translated proteins produced by TNT Coupled Reticulocyte Lysate System (Promega). The reactions were stopped by washing twice in

kinase buffer and boiling in 2 × SDS loading buffer. Proteins were resolved by 9% SDS-PAGE, and ³²P incorporation was detected by autoradiography. For *in vivo* labelling experiments, HeLa cells were transfected with the indicated plasmids for 24 h, and the medium was changed to phosphate-free DMEM with 0.5% dialysed FBS containing 200 μCi ml⁻¹ ortho-³²PO₄ for 4 h. Cells were lysed by RIPA buffer for immunoprecipitation and the immune complexes was subjected to 9% SDS-PAGE, followed by autoradiography analysis.

Flow cytometry- Cell cycle distribution was monitored by FACS analysis of ethanol fixed, propidium iodide stained cells on a Becton Dickinson FACS Calibur Analytical Flow Cytometer.

Statistical Analysis- All assays were repeated at least three times. The results of quantitative studies are reported as mean ± S.D. Differences were analyzed by Student's *t* test. *p* < 0.05 was regarded as significant, and such differences are indicated in the figures.

Results

Pim-1 stabilizes Skp2 protein. Overexpression in HeLa cells of wild type Pim-1 but not a kinase dead mutant, K67M, leads to a decrease in the level of the p27 protein (Fig. 1A) without any change in the mRNA level of this protein (Fig. S2D). To evaluate the mechanism by which Pim-1 functions, we focused attention on the E3 ligase SCF complex that targets p27 for proteasomal degradation, and in particular the Skp2 protein which is known to directly bind p27. Western blots demonstrate that transfection of the Pim-1 kinase increases the levels of Skp2 protein (Fig. 1A) while conversely siRNA or shRNA (Fig. S1A) knockdown of endogenous Pim-1 expression reduces Skp2 levels. The interplay between these two proteins is further demonstrated by the observation that transfection of murine Pim-1 into HeLa cells in which endogenous enzyme has been knocked down again elevates the level of Skp2 (Fig. 1A). Using two small molecule Pim kinase inhibitors, SMI-4a, which has demonstrated excellent selectivity (16-17,41), and a structurally unrelated Pim kinase inhibitor, K00135 (27), treatment of both HeLa cells (Fig. 1B) and PC3

prostate cancer cells (Fig. S1B, S1C) causes a dose-dependent reduction of Skp2 protein expression and a concomitant rise in p27. We have shown that Pim-1 facilitates cell cycle progression as overexpression of Pim-1 promotes G1-S transition (15) while Pim kinase inhibitor caused cell cycle arrest at G1 (16). Because the Akt protein kinase family is thought to control the level of Skp2 (28-29), we evaluated whether the PI3K inhibitor, wortmanin or a pan-Akt inhibitor, GSK690693 had similar affects on Skp2 levels. However, no significant changes in the levels of Skp2 were seen after treatment with these reagents until the highest concentrations tested (Fig. S1D, S1E). Interestingly, LY294002, which is both a PI3K and Pim-1 inhibitor (9), reduced Skp2 expression (Fig. S1E).

To test whether the effects of Pim-1 knockdown were cell cycle specific, we transfected HeLa cells with Pim-1 siRNA, blocked them in G1/S boundary, and then released them into cell cycle, and measured Skp2 and p27 levels. We find that the siRNA knockdown of Pim-1 regulated these two proteins throughout the cell cycle (Fig. 1C).

To test the activity of Pim-1 in a different cellular system we examined the role of Pim-1 overexpression in mouse prostate epithelial cells (MPECs). These cells respond to hepatocyte growth factor (HGF), a powerful mitogen and morphogen for epithelial and endothelial cells, through binding to its receptor the MET tyrosine kinase (42-43). The growth inhibitory activity of HGF on cancer cells is associated with upregulation of p27 expression (44), mediated by downregulation of Skp2 expression (45). We find that the HGF-induced p27 upregulation is inhibited by Pim-1 in MPECs (Fig. 1D). Similar results were also obtained in Pim overexpressing HeLa cells when they were treated with HGF (Fig. S1F).

Finally, in HEK293T cells the coexpression of Pim-1, but not kinase dead Pim-1, K67M or GFP, was able to induce a longer Skp2 half-life (Fig. 1E). Taken together, these experiments suggest that Pim-1 controls the levels of Skp2, and consequently regulates the amounts of p27 protein in cells.

Pim-1 directly binds to Skp2 and reduces Skp2 ubiquitination. We cotransfected HEK293 cells with Flag-Skp2 and either HA-Pim-1 or kinase dead Pim-1 (HA-K67M) expression constructs. When cell lysates were subjected to immunoprecipitation with HA antibody, we find that Pim-1 and Skp2 are able to physically interact in cells irrespective of the Pim-1 kinase activity (Fig. 2A). In HEK293T cells that are transfected, then serum starved, and finally released into 15% serum, this interaction between Pim-1 and Skp2 occurs maximally between hours 8 and 24 (Fig. 2B). We did not perform a cell cycle analysis on these cells. However, knockdown of endogenous Pim-1 in HeLa cells appears to reduce Skp2 expression throughout the cell cycle (Fig. 1C). These observations suggest that Pim-1 may regulate other molecule(s) controlling Skp2 levels *in vivo*. This binding is also seen *in vitro* in glutathione S-transferase (GST) pulldown experiments. Recombinant His-tagged Pim-1 protein binds to Skp2; the binding of Pim-1 did not interfere with the interaction between Skp2 and Ubc3, an E2 ubiquitin enzyme that is known to interact with the Skp2 protein (Fig. 2C). Furthermore, Pim-1 did not interfere with the formation of the SCF^{Skp2} complex, from Skp2, Ubc3 and Rbx1 proteins. (Fig. S2A).

Like p27, Skp2 levels are regulated by ubiquitination and proteasome degradation (25-26), suggesting that Pim-1 could decrease the levels of Skp2 ubiquitination. Using protein extracts from HEK293T cells transfected with Pim-1 and Skp2, we find that the presence of active but not kinase dead Pim-1 is sufficient to markedly repress the ubiquitination of the Skp2 protein (Fig. 2D). Using this same approach, consistent with the effect on Skp2, Pim-1 transfection was found to also increase p27 ubiquitination (Fig. S2C). This suggests that an increase in Skp2 levels is needed to mediate increased ubiquitination of p27 by Pim-1. Indeed, in an *in vitro* assay, the presence of Pim-1 did not directly influence p27 ubiquitination (Fig. S2B).

Pim-1 phosphorylates Skp2 on multiple sites. To determine whether Skp2 was a substrate for Pim-1, purified His-tagged protein kinase was incubated with immunoprecipitated Flag-Skp2 in the presence of [γ -³²P]-ATP. In this assay Skp2

was clearly phosphorylated and this phosphorylation was decreased by the addition of a small molecule Pim-1 inhibitor, SMI-4a (Fig. 3A). Pim-1 is known to phosphorylate the sequence R-X-R-L-S/T (46). Scanning the Skp2 sequence, we identified a potential Pim-1 consensus site at the carboxyl-terminal of Skp2, Thr417 (T417), which is conserved from frog to humans (Fig. 3B). Mutation of this residue from threonine to alanine (T417A) led to reduced Skp2 phosphorylation by Pim-1 *in vitro*, but did not completely abolish this modification (Fig. 3C). Previous studies (8-10) have demonstrated that Ser64 (S64) and Ser72 (S72) in Skp2 are CDK2 phosphorylation sites (27) and Ser72 can also be phosphorylated by Akt1 (28-29). Using GST-Pim-1 as a kinase, mutation of either Ser64 or Ser72 to Ala markedly decreased Skp2 phosphorylation by Pim-1 with both of these changes had a somewhat greater effect than the T417A mutation (Fig. 3D), suggesting that each of these sites might also be a Pim-1 target. Phosphorylation of Ser64 and/or Ser72 may be required for phosphorylation on Thr417 since mutation of either Ser64 or Ser72 almost completely abolished Skp2 phosphorylation. A complete understanding of the control of phosphorylation of these sites requires further study.

To test whether Pim-1 has a role in regulating Skp2 phosphorylation *in vivo*, HeLa cells were transfected with Pim-1 and Skp2, metabolically labeled with orthophosphate, and then treated with kinase inhibitors such as roscovitine (pan-CDK inhibitor), wortmannin (PI3K inhibitor), and SMI-4a (Pim-1 inhibitor). Treatment with roscovitine and wortmannin reduced Skp2 phosphorylation *in vivo*. Overexpression of Pim-1 markedly increased Skp2 phosphorylation, and this phosphorylation was inhibited by all three agents (Fig. 3E), suggesting that multiple kinases and kinase pathways play a role in regulating phosphorylation of this protein.

Skp2 is degraded by the APC/C^{Cdh1} (25-26) which is known to have its highest activity from late mitosis to the G1 phase of the cell cycle (47). To test the impact of phosphorylation of Skp2 on protein stability, we used HeLa cells that were released from a thymidine-nocodazole block in the G1 phase of cell cycle into media containing

cycloheximide. Exit from mitosis was monitored by the loss of histone H3 phospho-Ser10 immunoreactivity and the degradation of cyclin B1 on Western blots (Fig. 3F). Using this technique, we find that all three individual Skp2 phosphorylation mutants were more efficiently degraded than the wild-type Skp2 protein (Fig. 3F), suggesting that phosphorylation by protein kinases, including Pim-1, controls the rate of degradation of Skp2.

We next examined the biological activity of wild type and Skp2 phosphorylation mutants by transfecting them along with p27 into HeLa cells, and then examining p27 levels by Western blot. We find that both the Skp2 T417A and S64A mutants decreased the ability of Skp2 to stimulate the degradation of p27 with T417A retaining some degrading activity (Fig. 3G). In contrast an aspartate mutation, T417D that mimics phosphorylation at this site was more efficient than the T417A at degrading p27. Surprisingly, S72A mutation, which has been suggested to regulate Skp2 activity (28-29) did not cause any detectable effect on p27 degradation (Fig. 3G).

Pim-1 impairs Cdh1 and CDC27 interaction, and phosphorylates Cdh1. Because Pim-1 regulates Skp2 ubiquitination we examined whether this enzyme might interact with components of the APC/C complex that are responsible for Skp2 degradation. In coimmunoprecipitation experiments done in transfected HEK293T cells, Pim-1 was found to complex with either Cdh1 or CDC20, two well-known activators of APC/C (Fig. S3A). However, Pim-1 did not physically impair the interaction between Cdh1 or CDC20 and Skp2 (Fig. S3B). Using the same methodology, in contrast, we find that Pim-1 could impair the interaction between Cdh1 and CDC27, another APC/C component, in a phosphorylation dependent manner (Fig. 4A). The two kinase dead Pim-1 mutants, K67M and NT81(36) are also able to form a complex with Cdh1, but only wild-type Pim-1 was capable of reducing the interaction between Cdh1 and CDC27 (Fig. 4A). Additionally, incubation with the Pim-1 inhibitor, SMI-4a (Fig. S4C) or treatment with siRNA to knock down endogenous Pim-1 expression (Fig. 4B) increased Cdh1/CDC27 interaction.

Our results are consistent with the previous findings that demonstrate that phosphorylation of Cdh1 dissociates this protein from APC/C complex (48-49). Cdh1 is hyperphosphorylated *in vivo* during S, G2, and M phase and this phosphorylation causes an electrophoretic mobility shift on SDS-PAGE gels (Fig. 5A, (48,50-51)). To further explore the role of Pim-1 in phosphorylation of Cdh1, we first reprobated the same membrane used in Fig. 1C with antibodies against Cdh1 and CDC27. In cells treated with Pim-1 siRNA, Cdh1 displayed higher mobility at 0, 4, 8, and 12 hrs compared to those in control cells (Fig. 1C), suggesting phosphorylation was reduced. To confirm this finding, we knocked down endogenous Pim-1 expression in HeLa cells using shRNA and then subjected these cells to a double-thymidine block. The cells were then released from the block (for cell cycle analysis, see Fig. 5A) and immunoblotting was performed to examine Cdh1 phosphorylation. Phosphorylation of endogenous Cdh1 at 8 h after release from a double-thymidine block was dramatically reduced in Pim-1 knockdown cells as judged by the protein mobility shift (Fig. 4C).

Additionally, we find that recombinant Pim-1 was capable of phosphorylating *in vitro* translated Cdh1 but not CDC20 (Fig. 4D). Cdh1 is heavily phosphorylated by CDKs *in vivo*. To examine whether Pim-1 can also phosphorylate Cdh1 *in vivo*, we first treated HeLa cells with the CDK inhibitor roscovitine and then labeled them with ³²P-orthophosphate. Roscovitine treatment reduced Cdh1 phosphorylation, and overexpression of Pim-1 in the presence of this reagent increased phosphorylation (Fig. 4E).

To examine whether Pim-1 has *in vivo* activity when expressed at endogenous levels, we treated HeLa cells with SMI-4a and found that this compound reduced Cdh1 phosphorylation (Fig. 4E). Because the APC/C activity to degrade Skp2 can be activated by Cdh1 overexpression (25-26), we tested the ability of Pim-1 to reverse this effect. We find that co-expression of wild-type Pim-1, but not its mutant, K67M, is capable of blocking Cdh1 mediated degradation of Skp2 (Fig. 4F).

Pim-1 is required for Skp2 to signal cell cycle S-phase entry. Based on the activities of Pim-1, we

have attempted to correlate the levels of this enzyme with other cell cycle regulatory components. HeLa cells were released from a double-thymidine block, cell cycle progression was monitored by FACS analysis (Fig. 5A), and the expression patterns of Skp2, p27, Cdh1, CDC27, cyclin B1, and cyclin A were measured by Western blots (Fig. 5B). We find that Pim-1 levels were very high at S (4 h) and G2/M phase (8 h) of the cell cycle. Lower Pim-1 expression was seen at G1 and G1/S boundary (0, 16, 20, and 24 h, Fig. 5A and 1C). Since Pim-1 is a constitutively active kinase (7-11), this expression pattern of Pim-1 should represent its activity profile during cell cycle progression. Interestingly but not surprisingly, Pim-1 activity coincides with Skp2 expression (Fig. 5A) and inversely correlates with Cdh1 activity (47,52) during cell cycle (Fig. 5A). Because Skp2 is known to have the ability to induce S phase in quiescent fibroblasts (40,53), we determined whether the Skp2-Pim-1 interaction is important for S phase progression. Investigation of this question was carried out using Rat1 cells, because they were able to undergo complete cell cycle blockade at G0/G1 upon serum starvation. As judged by FACS analysis, we find that in the absence of serum addition, over-expressed Skp2 or Pim-1 each stimulates S phase entry (Fig. 5B), and coexpression of Skp2 and Pim-1 further enhanced the S phase entry of these cells (Fig. 5B). Conversely, treatment with a small molecule Pim inhibitor, SMI-4a reduced Skp2 induced S phase progression (Fig. 5C) and impaired serum-induced S phase entry (Fig. 5C). Another structurally unrelated small molecule Pim inhibitor, K00135 (54) displayed a similar effect (Fig. S5A). These observations suggest that Pim kinases are required for maximal Skp2 activity to allow cells to exit from quiescence.

Discussion

The data presented suggests the novel observation that the Pim-1 protein kinase through a dual mechanism can regulate the levels and hence the activity of Skp2 (see model in Figure 6). Pim-1 is capable of binding and phosphorylating Skp2 and stabilizing protein levels, but does not affect the interaction of Skp2 with the E2 ligase Ubc3. Conversely, both siRNA and small molecule Pim-1 inhibitors decrease Skp2 levels and

phosphorylation. Skp2 is phosphorylated by CDK2 at Ser64 and Ser72 (27), and by Akt1 at Ser72 to stabilize this protein (28-29). Pim-1 appears capable of phosphorylating Skp2 at these two sites (Fig. 3), as well as a unique site in the carboxy terminus, Thr417, that is highly conserved throughout the animal kingdom, including humans and mouse. Phosphorylation of this site is required for maximal Skp2 activity and stabilization of Skp2 protein levels *in vivo* (Fig. 3). In the prostate cancer cell line PC3 that contains an activated Akt a small molecule Pim inhibitor SMI-4a but not wortmannin or the Akt inhibitor, GSK690693 decreased the levels of Skp2. LY294002, which inhibits both Akt and Pim, displayed a similar effect to SMI-4a, suggesting that in this cell line the Pims are essential for the regulation of Skp2 levels. Unlike Akt (28-29), Pim-1 kinase did not appear to regulate Skp2 subcellular localization (Fig. S4). The Pim kinases share multiple similarities with AKT (1,55-56). It is possible that the relative abundance of each of these Skp2 phosphorylating kinases may decide which is essential to the control of Skp2 levels. In our hands the Skp2 S72A mutant did not lose p27 degradation activity compared to the wild-type Skp2 (Fig. 3G). Although previously reported to regulate ubiquitin ligase activity (28-29) two recent reports confirmed our finding (30-31). The half-life of this mutant was shorter than that of wild-type Skp2 (Fig. 3F) consistent with previous reports (27-29).

The degradation of Skp2 is regulated by APC/C^{Cdh1} complex (25-26) which preferentially associates with non-phospho-Ser64 form of Skp2 (27). Pim-1 kinase activity does not affect (Figs. 2C and S2A) the binding of Cdh1 to total Skp2 (Fig. S4B), but does impair the interaction between Cdh1 and CDC27 (Figs. 4A, and S4C). Interaction with CDC27/APC3 protein allows Cdh1 to activate the APC/C (57). Although Cdh1 is inhibited by both the Emi1 protein and multiple

phosphorylations initiated in part by cyclin A-CDK2 and cyclin B1-CDK1 (47,52), it has been proposed that an additional kinase may play a role (58). We demonstrate that Cdh1 is a phosphorylation target of Pim-1 (Figs. 4D and 4E) and that the knockdown of Pim-1 with siRNA reduces Cdh1 phosphorylation during S, G2, and M phases (Figs. 1C and 4C) demonstrating the critical involvement of Pim-1 in tightly controlled Cdh1 phosphorylation during the cell cycle. It is not known whether Pim-1 and CDKs share identical phosphorylation sites on Cdh1. Further studies are required to determine the precise Pim-1 sites and how these two different kinases cooperate to control Cdh1 activity. The levels of Pim-1 protein are correlated with Cdh1 phosphorylation during cell cycle progression as high Pim-1 expression and high Cdh1 phosphorylation were seen during S, G2, and M phases, and the opposite occurred during the G1 phase (Fig. 5A). Given the role of Cdh1 in regulating mitosis, this may explain why Pim-1 is not only required for Skp2 to signal S phase entry (Fig. 5), but also plays a critical role in G2/M phase regulation. Consistent with this hypothesis, mouse embryo fibroblasts that are knock-out for all three Pim kinase isoforms displayed increased number of cells in the G2/M phase of the cell cycle (Fig. S5). These observations are in concert with previous discoveries suggesting that Pim-1 functions in mitosis (59-61). Therefore, the Pim-1 kinase regulates Skp2 levels by reducing APC/C^{Cdh1} E3 ligase activity and thus protects Skp2 from degradation (Fig. 4F).

The Pim-1 protein kinase is abnormally elevated in human cancers, regulated by growth factors, and collaborates with other oncogenes to induce cell transformation (1-2,5-6). The ability of this enzyme to modulate the activity of both the SCF^{Skp2} and APC/C^{Cdh1} (Fig. 6) and thus control p27 levels is likely to be essential to the biological activities of this protein kinase.

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Footnotes:

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The abbreviations used are: APC/C, anaphase-promoting complex or cyclosome; C-TAK1, Cdc25C-associated kinase 1; HGF, hepatocyte growth factor; GST, glutathione *S*-transferase; SCF, Skp1/cullin/F-box protein; PSA, prostate specific antigen.

Figure Legends

Fig. 1. Regulation of Skp2 protein levels by Pim-1. (A) HeLa cells were transiently transfected with cDNAs encoding green fluorescent protein (GFP), Pim-1, kinase dead Pim-1 (K67M), or a siRNA to Pim-1 together with GFP, or Skp2, or Pim-1, or a scrambled sequence. Forty-eight hours after transfection extracts of these cells were probed on western blots with the listed antibodies. (B) HeLa cells were treated with various concentrations of Pim kinase inhibitor SMI-4a for 16 h, extracts prepared, and immunoblots carried out with the identified antibodies. (C) HeLa cells were transfected with the indicated siRNA plasmids followed by a double thymidine block treatment (see Methods and Materials). Lysates were prepared at the indicated time points after release from the thymidine block, and subjected to immunoblot analysis. Arrows indicate phosphorylated and unphosphorylated forms of Cdh1. (D) Mouse prostate epithelial cells (MPECs) stably transfected with a control vector (pLNCX) or a human Pim-1 expressing plasmid were treated with HGF (50 ng/ml) for 24 h followed by immunoblot analysis. (E) Twenty-four hours after transfection with expression plasmids (= time 0), HEK293T cells were incubated for the indicated times with cycloheximide (CHX, 100 μ g/ml) followed by immunoblot analysis with FLAG or β -actin antibodies. Densitometric analysis was performed using National Institutes of Health ImageJ software to quantify the expression of Skp2. Skp2 band intensity was first normalized to β -actin, and then normalized to the t=0 controls.

Fig. 2. Pim-1 binds to Skp2. (A) HEK293T cells were transfected with the indicated plasmids, protein immunoprecipitated (IP) with HA antibody (Ab) and immunoblotted with flag or Pim-1 Ab. Lysates of cells used for this assay were probed with identical Abs. (B) HEK293T cells were serum starved (0.2%) for 48 h prior to addition of 15% FBS at 0 time. Cells were then harvested at the indicated time points and coimmunoprecipitation (co-IP) preformed. (C) GST-Skp2 proteins were incubated overnight with his tagged Pim-1 or Ubc3 proteins purified from *E. coli* at 4 °C, washed with PBS, and subjected to immunoblot analysis (upper panel). GST and GST-Skp2 were stained with Coomassie brilliant blue (lower panel). (D) HEK293T cells were transfected with the indicated plasmids, treated with 10 μ M MG132 for 6 h, and ubiquitination measured by binding to Ni⁺ beads (Materials and Methods) followed by an immunoblot with anti-HA ab. Immunoblot analysis was performed on total cell lysates from these HEK293T cells (two lower panels).

Fig. 3. Pim-1 phosphorylates Skp2. (A) FLAG-Skp2 was immunoprecipitated from HEK293T cells, incubated with recombinant His-tagged Pim-1 for 30 min with or without SMI-4a for an *in vitro* kinase assay (Materials and Methods) was carried out followed by SDS-PAGE autoradiography (upper panel), and immunoblot analyses (lower panel). (B) The carboxyl terminus sequence of Skp2 contains a Pim-1

consensus site (C) GST-tagged Skp2 proteins or a T417A mutant were incubated with recombinant GST-Pim-1 and [γ - 32 P]-ATP for 30 min, and subjected to SDS-PAGE followed by autoradiography. (D) Wild type FLAG-Skp2 and its mutants T417A, S64A, S72A, and S64A/S72A were immunoprecipitated from HEK293T cells, incubated with recombinant GST tagged Pim-1 and [γ - 32 P]-ATP for 30 min, followed by SDS-PAGE autoradiography (upper panel) and immunoblot analysis (lower panel). (E) HeLa cells were pretreated with roscovitine (20 μ M), wortmannin (1 μ M), or SMI-4a (10 μ M) for 1h transfected with human Pim-1 and Skp2 and labeled with 32 P-orthophosphate followed by Flag immunoprecipitation, autoradiography (upper panel) and Flag/Pim-1 immunoblots (two lower panels). (F) HeLa cells were transfected with the indicated Skp2 constructs and synchronized in M phase by mitotic shake-off of cells obtained after release from a thymidine-nocodazole block. The cells were then replated and allowed to progress through the cell cycle in the presence of cycloheximide (100 μ g/ml). Immunoblot analysis was performed at specific time points using Ab to cyclin B and phospho Histone H3 Ser10 (p-H3 (S10) as controls. Densitometric analysis was performed using National Institutes of Health ImageJ software to quantify the expression of Skp2. Skp2 band intensity was normalized to β -actin, then normalized to the t=0 controls. (G) HEK293T cells were transfected with a FLAG-tagged p27 Skp2 constructs or a GFP control. Expression of exogenous p27 and Skp2 is measured by immunoblotting.

Fig. 4. Pim-1 kinase phosphorylates Cdh1 and impairs its binding to CDC27 (A) HEK293T cells were transfected with HA-Cdh-1, Pim-1 or kinase dead Pim-1 K67M or NT81, immunoprecipitated with HA Ab followed by Western blotting with Abs to CDC27 and Pim-1. Lysates from these cells were immunoblotted with Ab as shown (B) HeLa cells were co-transfected with HA-Cdh1 and Pim-1 or scrambled siRNA plasmids before harvesting for co-immunoprecipitation analysis. Levels of transfected proteins in lysates were monitored by immunoblot. (C) HeLa cells were transfected with the indicated shRNA plasmids followed by a double thymidine block. After release from the block cell, lysates were prepared at 8 and 16h, and subjected to immunoblot analysis. The arrows indicate phosphorylated and unphosphorylated Cdh-1 (D) *In vitro* translated HA-tagged Cdh1 or Cdc20 was incubated with recombinant Pim-1 and [γ - 32 P]-ATP in an *in vitro* kinase assay. Autoradiography (upper panel) and immunoblot (lower panel) analysis was performed. (E) HeLa cells were transfected with HA-Cdh1 and human Pim-1 and the kinase inhibitors roscovitine (20 μ M) or SMI-4a (10 μ M) were added 1h before labeling with 32 P-orthophosphate. HA-Cdh-1 was immunoprecipitated and autoradiography (upper panel) and immunoblot analysis (lower panel) performed (F) HEK293T cells were transfected with Flag-Skp2, myc-Cdh-1 and HA tagged WT and kinase dead (K67M) Pim kinase and the extracts immunoblotted with the specified antibodies.

Fig. 5. Pim-1 is required for Skp2 to signal cell cycle S phase entry. (A) HeLa cells were treated with a double thymidine block and released into fresh media. Cells were then harvested at the indicated time points and subjected to FACS (left panel) and immunoblot analysis (right panel). The arrow denotes the Pim-1 signal. *indicates a nonspecific signal. (B) Rat1 cells were transduced with a lentivirus carrying indicated cDNAs. Cells were maintained in low serum conditions (0.2%) for 48 h before harvested for FACS (upper panel) and immunoblot (lower panel) analyses. The percentage of S phase cells was compared to vector control, except where indicated by a bracket. (C) The experiment was performed as in (B) except that SMI-4a (5 μ M) was added 3 h before a 20% FBS stimulation (16 h). Brackets indicate a comparison of with and without SMI-4a treatment.

Fig. 6. A model of depicting Pim-1 regulation of Skp2 degradation. Nonphosphorylated Skp2 binds to E3 ligase APC/C^{Cdh1} and is ubiquitinated followed by proteasome-mediated degradation. Pim-1 kinase phosphorylates both Skp2 and Cdh1 reducing Cdh1 and APC/C interaction, decreasing Skp2 ubiquitination, and consequently increasing Skp2 stability.

Figure 1

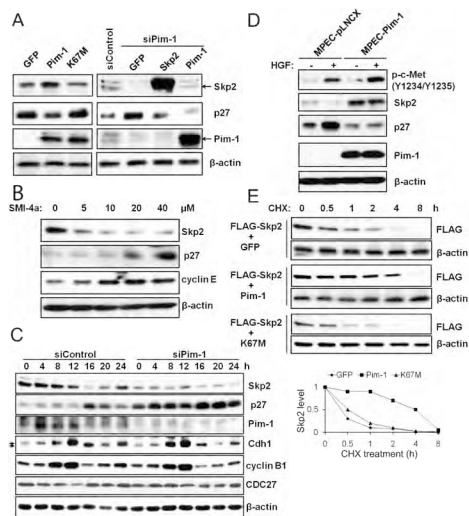


Figure 2

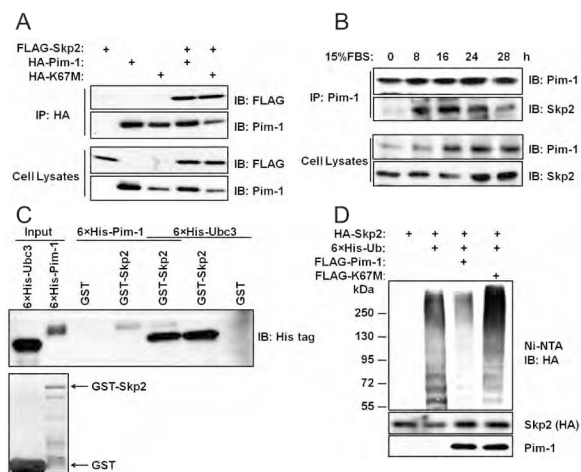


Figure 3

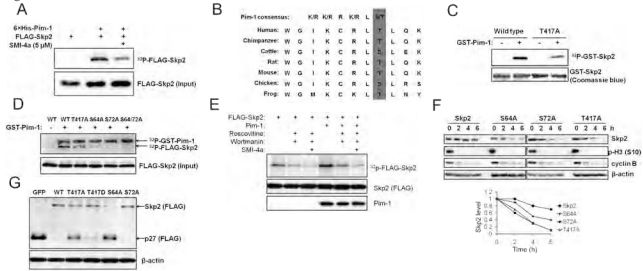


Figure 4

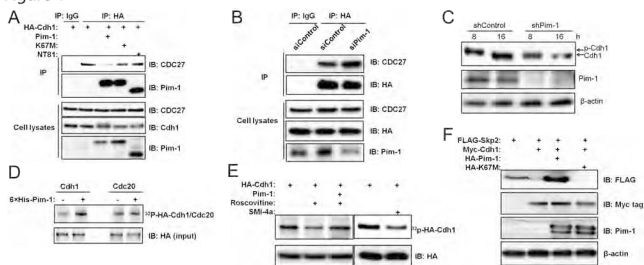


Figure 5

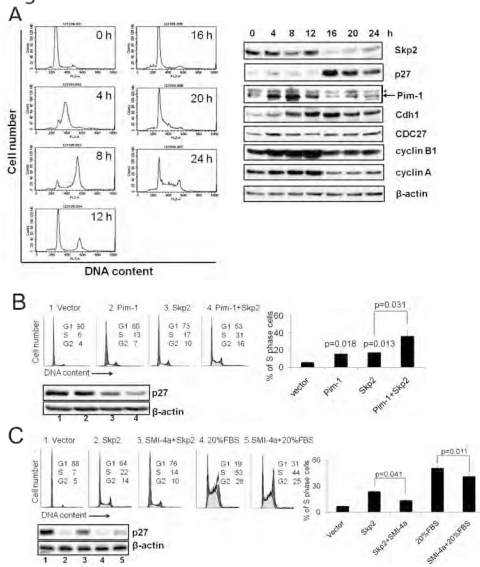
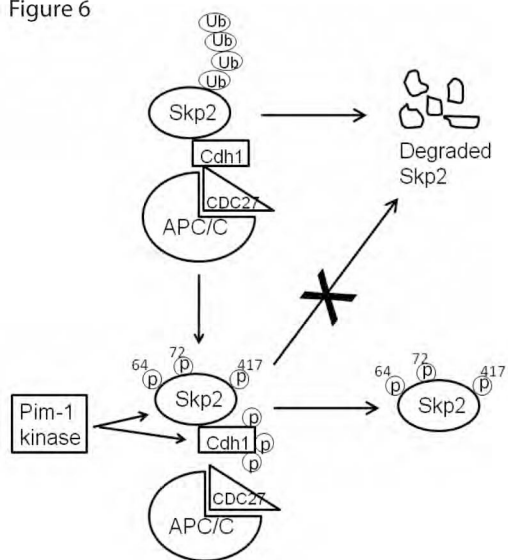


Figure 6



Supplemental Figure Legends

Fig. S1. Pim-1 positively regulates Skp2 protein expression. (A) HeLa cells were transfected with the indicated pGIPZ shRNA constructs and harvested for immunoblot analysis with Ab to Skp2, Pim-1 with β -actin as a loading control. (B) Immunoblot analysis of PC3 human prostate cancer cells treated with the indicated concentrations of Pim kinase inhibitor SMI-4a for 16 h. (C) Immunoblot analysis of PC3 cells treated with the indicated concentrations of Pim kinase inhibitor K00135 for 16 h. (D) Immunoblot analysis of PC3 cells treated with the indicated concentrations of Pim kinase inhibitor SMI-4a or PI3K inhibitor wortmannin for 16 h. (E) Immunoblot analysis of PC3 cells treated with the indicated concentrations of PI3K inhibitor LY294002 or AKT inhibitor GSK690693 for 16 h. (F) HeLa cells were transfected with a plasmid encoding GFP or human Pim-1 and cells were treated with HGF (50 ng/ml) for 24 h. Immunoblot analysis was performed on the extracts.

Fig. S2. Pim-1 does not affect SCF^{Skp2} activity *in vitro*. (A) HEK293T cells were transfected with the indicated plasmids and extracts harvested to examine co-immunoprecipitation. (B) *In vitro* translated p27 was labeled with ³⁵S and mixed with additional components to measure *in vitro* ubiquitination (see Experimental Procedures). Phosphor imaging was performed on the resulting SDS-PAGE. (C) HEK293T cells were transfected with the indicated plasmids, treated with 10 μ M MG132 for 6 h, extracted harvested, and ubiquitination measured by immunoblotting. (D) Total mRNA was extracted from HeLa cells transfected with GFP or Pim-1 and this mRNA was subjected to real-time PCR analysis.

Fig. S3. Pim-1 does not impair Skp2 interaction with Cdh1 or Cdc20. (A) HEK293T cells were transfected with the indicated plasmids and harvested for co-immunoprecipitation assay (upper panel). The lower panel demonstrates the level of proteins in these cell lysates. (B) This experiment was carried out similarly to (A) with the upper panel demonstrating immunoprecipitations and the lower panel showing an immunoblot of the same extracts. (C) HeLa cells were transfected with HA-Cdh1 and treated with DMSO or SMI-4a (10 μ M, 24 h) before harvesting for co-immunoprecipitation. The upper panel contains the immunoprecipitation and the lower one immunoblots of identical extracts.

Fig. S4. Pim-1 does not regulate Skp2 subcellular localization. HeLa cells were transfected with the expression vectors (panel A) or siRNAs (panel B), cells harvested, and nucleus fraction (N) and cytoplasm fraction (C) separated by standard subcellular fractionation techniques (1). Extracts of cells were made and probed with Abs to Skp2, Pim-1 and Lamin B as a nuclear marker and β -tubulin to denote cytoplasm. Densitometric analysis was performed using National Institutes of Health ImageJ software to quantify the expression of Skp2 protein normalized to β -tubulin (cytoplasm) and Lamin B (nucleus) expression, respectively. Results are shown as means \pm s.d. for three independent experiments.

Fig. S5. Effect of Pim kinases on cell cycle distribution. (A) The experiment was performed as in Fig. 5C except that K00135 (10 μ M) was used instead of SMI-4a. Brackets indicate comparison of with and without K00135 treatment. (B) FACS analysis was performed on wild-type (WT) and Pim-1,2, 3 triple-knockout (TKO) mouse embryonic fibroblasts (2) 50% confluent growing in 10% FBS and DMEM media.

Supplemental References

1. Nikolaev, A. Y., Li, M., Puskas, N., Qin, J., and Gu, W. (2003) *Cell* **112**, 29-40
2. Mikkers, H., Nawijn, M., Allen, J., Brouwers, C., Verhoeven, E., Jonkers, J., and Berns, A. (2004) *Mol Cell Biol* **24**, 6104-6115

Figure S1

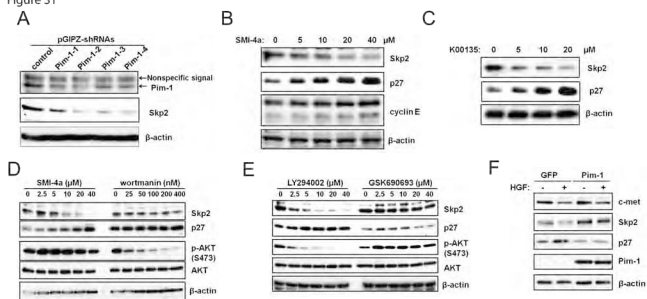


Figure S2

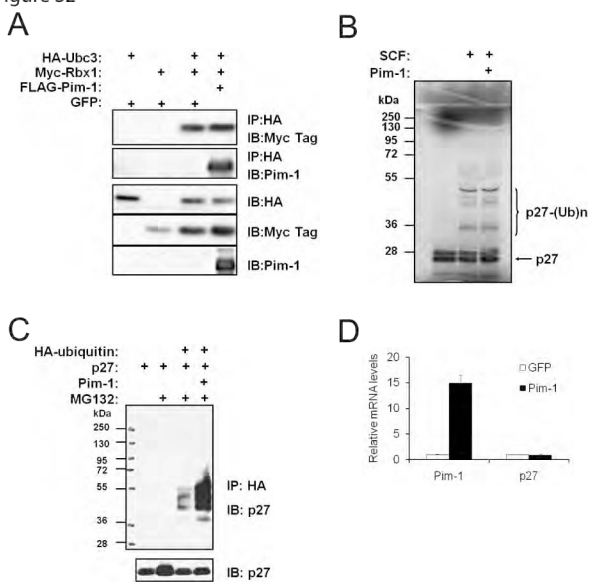


Figure S3

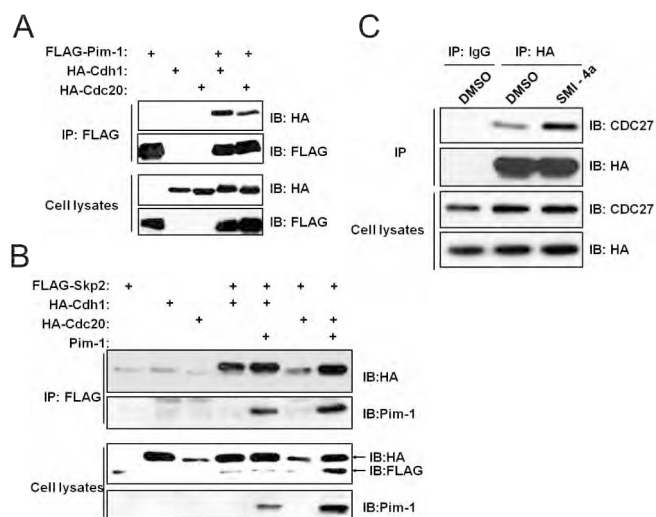


Figure S4

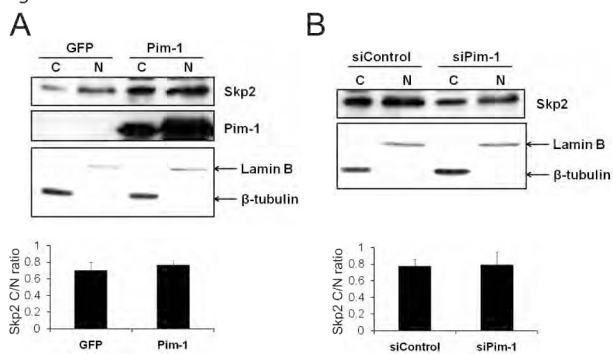
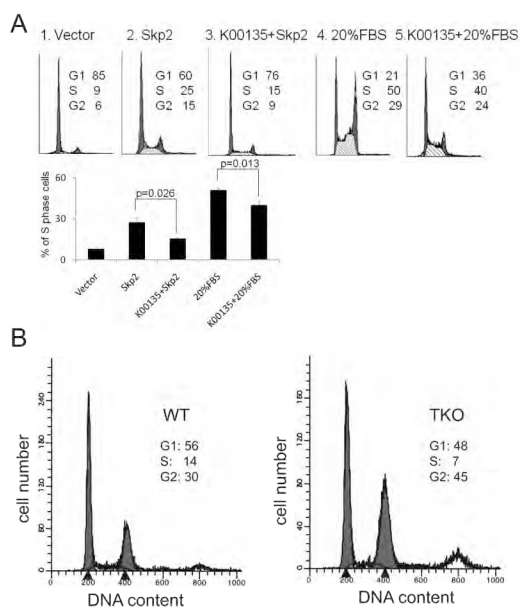


Figure S5



SUPPORTING DATA

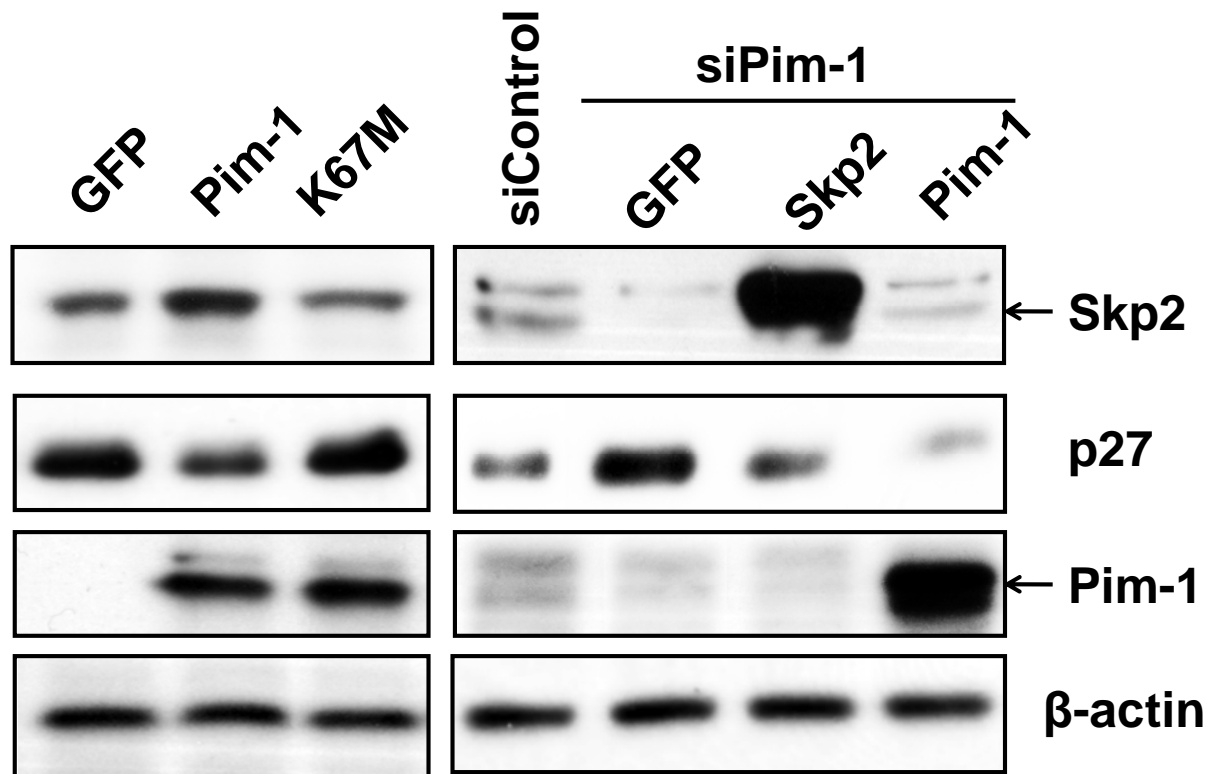


Fig. 1: HeLa cells were transiently transfected with cDNAs encoding green fluorescent protein (GFP), Pim-1, kinase dead Pim-1 (K67M), or a siRNA to Pim-1 together with GFP, or Skp2, or Pim-1, or a scrambled sequence. Forty-eight hours after transfection extracts of these cells were probed on western blots with the listed antibodies.

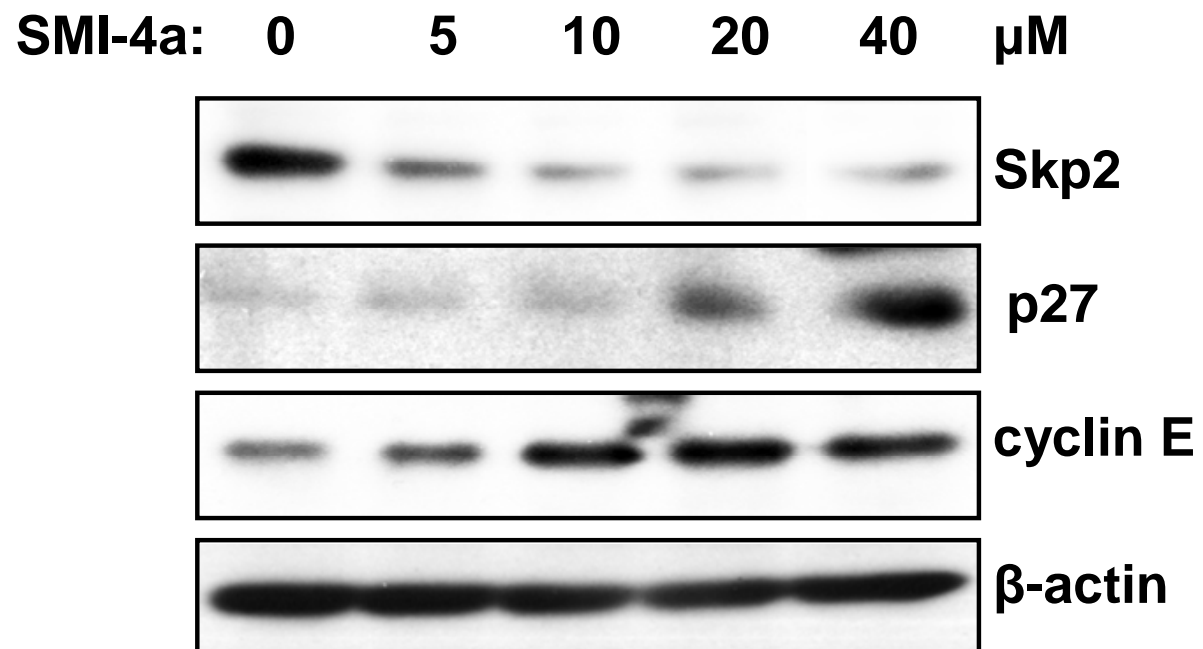


Fig. 2: HeLa cells were treated with various concentrations of Pim kinase inhibitor SMI-4a for 16 h, extracts prepared, and immunoblots carried out with the identified antibodies.

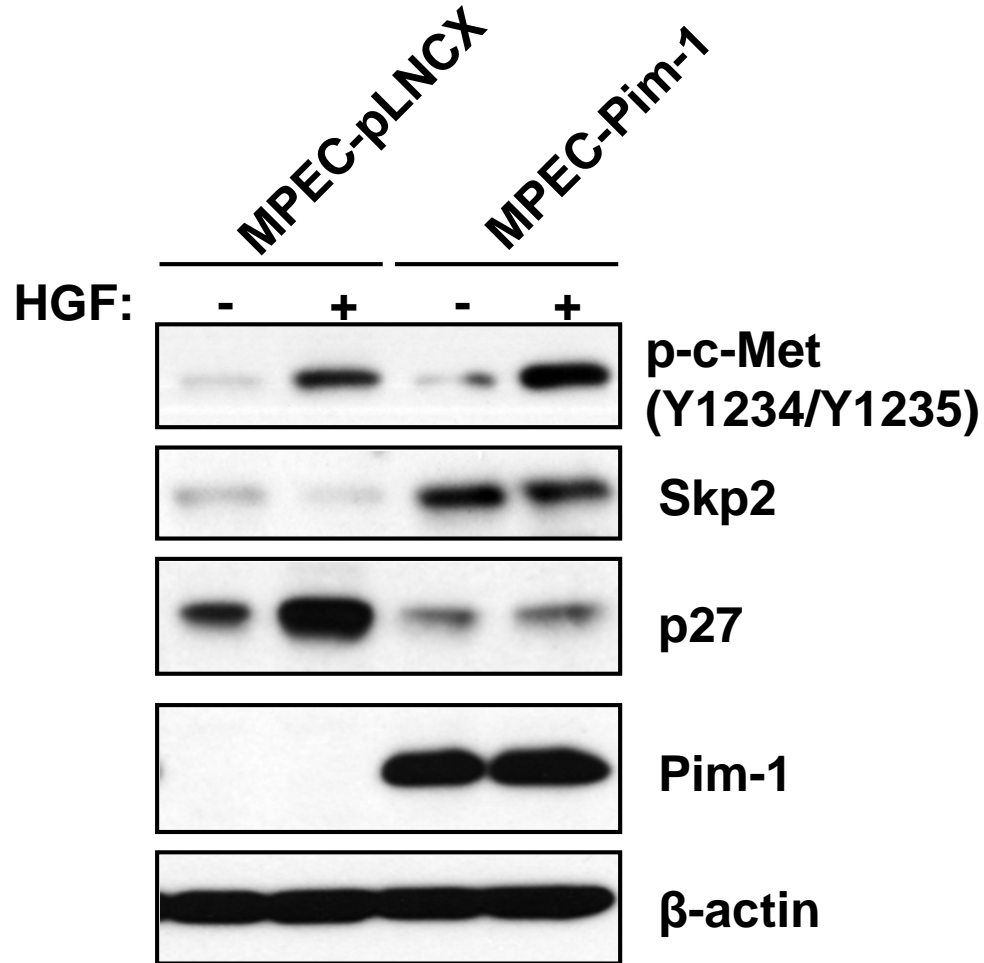


Fig. 3: Mouse prostate epithelial cells (MPECs) stably transfected with a control vector (pLNCX) or a human Pim-1 expressing plasmid were treated with HGF (50 ng/ml) for 24 h followed by immunoblot analysis.

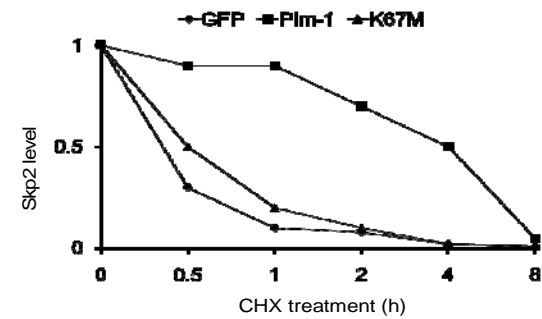
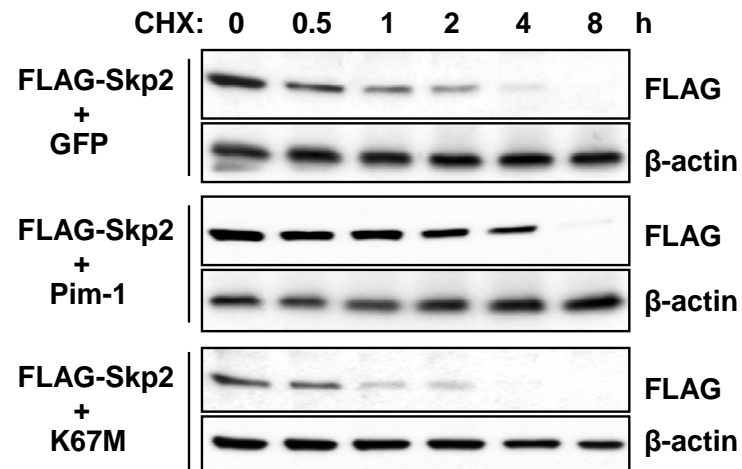


Fig. 4: Twenty-four hours after transfection with expression plasmids (= time 0), HEK293T cells were incubated for the indicated times with cycloheximide (CHX, 100 μ g/ml) followed by immunoblot analysis with FLAG or β -actin antibodies. Densitometric analysis was performed using National Institutes of Health ImageJ software to quantify the expression of Skp2. Skp2 band intensity was first normalized to β -actin, and then normalized to the t=0 controls.

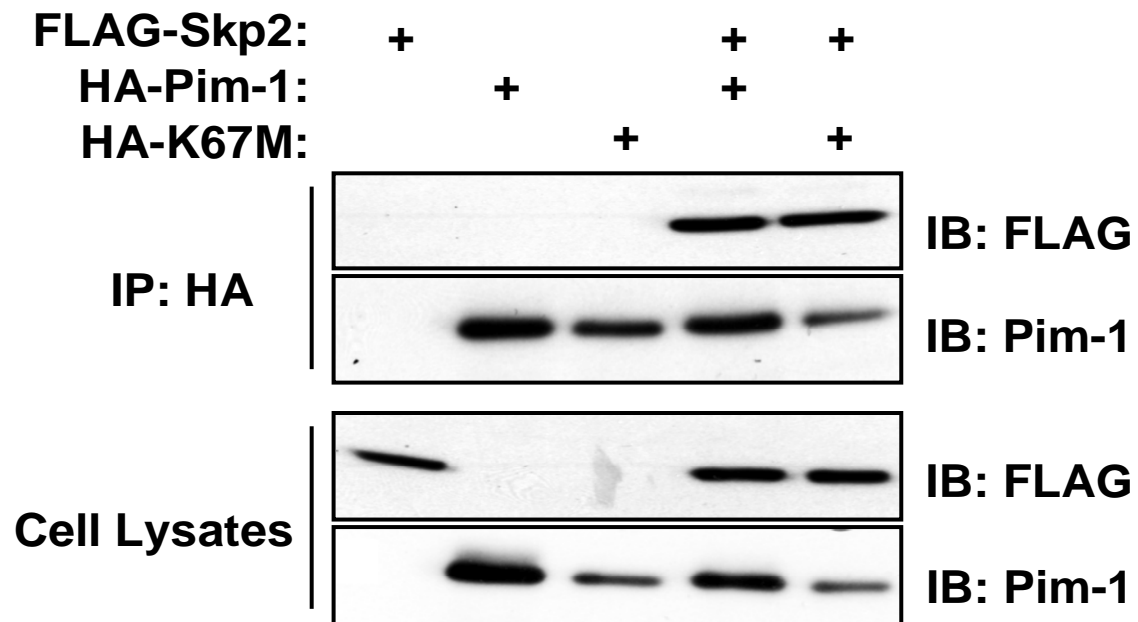


Fig. 5: HEK293T cells were transfected with the indicated plasmids, protein immunoprecipitated (IP) with HA antibody (Ab) and immunoblotted with flag or Pim-1 Ab. Lysates of cells used for this assay were probed with identical Abs.

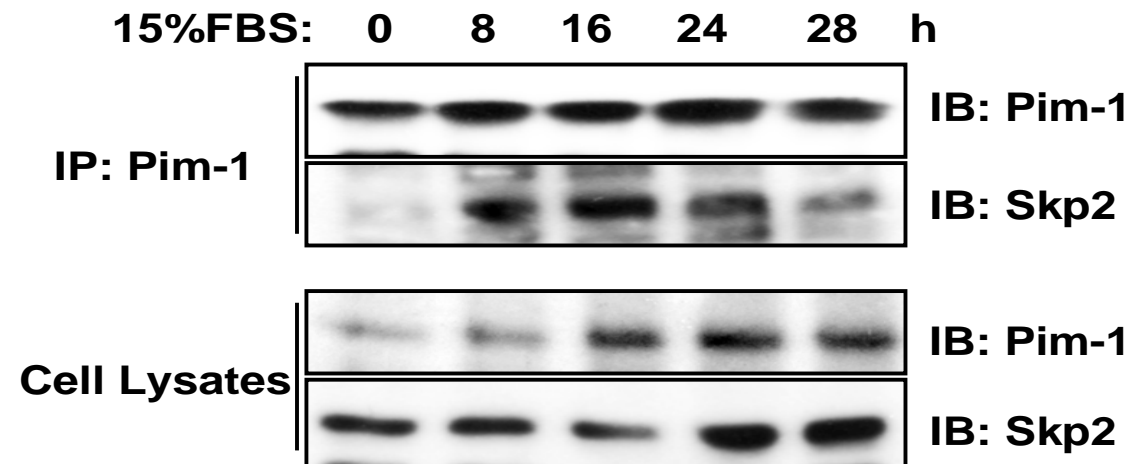


Fig. 6: HEK293T cells were serum starved (0.2%) for 48 h prior to addition of 15% FBS at 0 time. Cells were then harvested at the indicated time points and coimmunoprecipitation (co-IP) preformed.

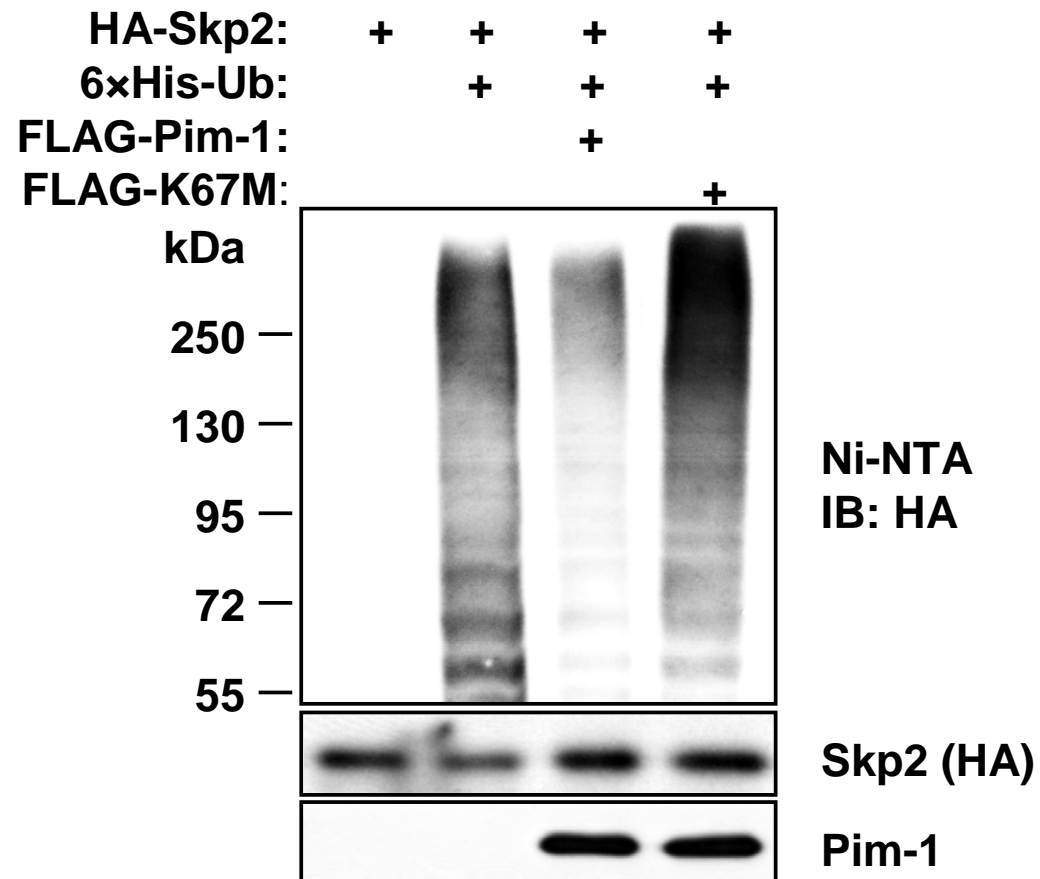


Fig. 7: HEK293T cells were transfected with the indicated plasmids, treated with 10 μ M MG132 for 6 h, and ubiquitination measured by binding to Ni²⁺ beads (Materials and Methods) followed by an immunoblot with anti-HA ab. Immunoblot analysis was performed on total cell lysates from these

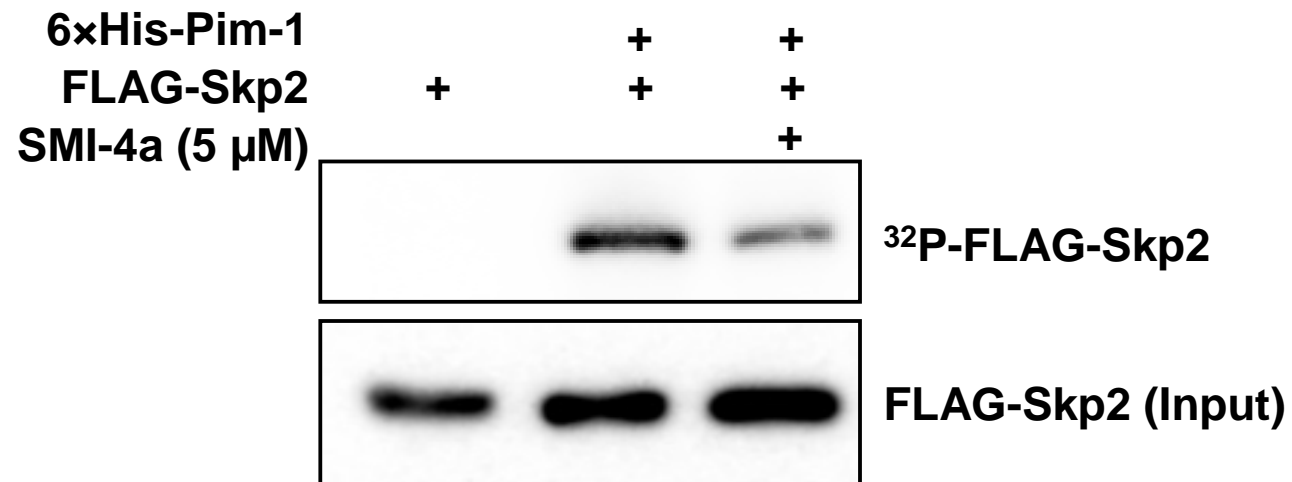


Fig. 8: FLAG-Skp2 was immunoprecipitated from HEK293T cells, incubated with recombinant His-tagged Pim-1 for 30 min with or without SMI-4a for an in vitro kinase assay (Materials and Methods) was carried out followed by SDS-PAGE autoradiography (upper panel), and immunoblot analyses (lower panel).

Pim-1 consensus:			K/R	K/R	R	K/R	L	S/T			
Human:	W	G	I	K	C	R	L	T	L	Q	K
Chimpanzee:	W	G	I	K	C	R	L	T	L	Q	K
Cattle:	W	G	I	K	C	R	L	S	L	E	K
Rat:	W	G	I	K	C	R	L	T	L	Q	K
Mouse:	W	G	I	K	C	R	L	T	L	Q	K
Chicken:	W	G	I	K	C	R	L	S	L	R	S
Frog:	W	G	M	K	C	K	L	T	L	N	Y

Fig. 9: The carboxyl terminus sequence of Skp2 contains a Pim-1 consensus site

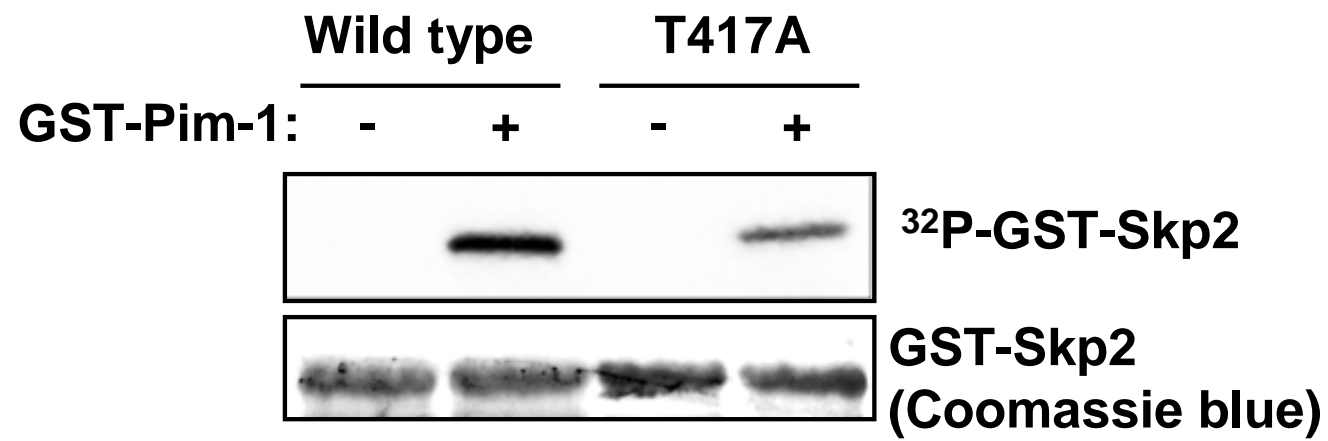


Fig. 10: GST-tagged Skp2 proteins or a T417A mutant were incubated with recombinant GSTPim-1 and [γ -³²P]-ATP for 30 min, and subjected to SDS-PAGE followed by autoradiography.

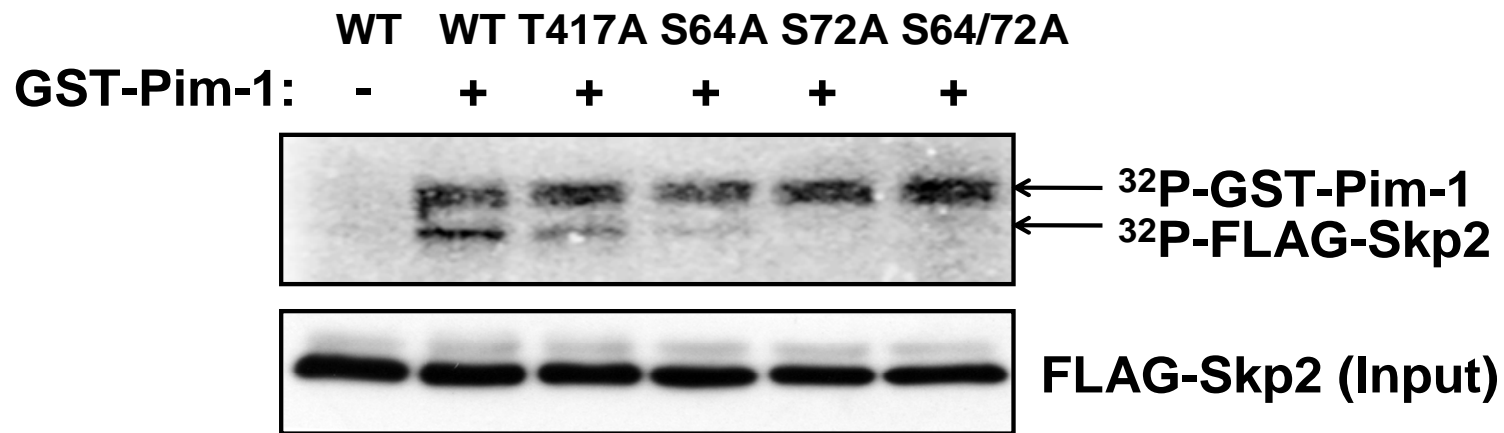


Fig. 11: Wild type FLAG-Skp2 and its mutants T417A, S64A, S72A, and S64A/S72A were immunoprecipitated from HEK293T cells, incubated with recombinant GST tagged Pim-1 and [γ - ^{32}P]-ATP for 30 min, followed by SDS-PAGE autoradiography (upper panel) and immunoblot analysis (lower panel).

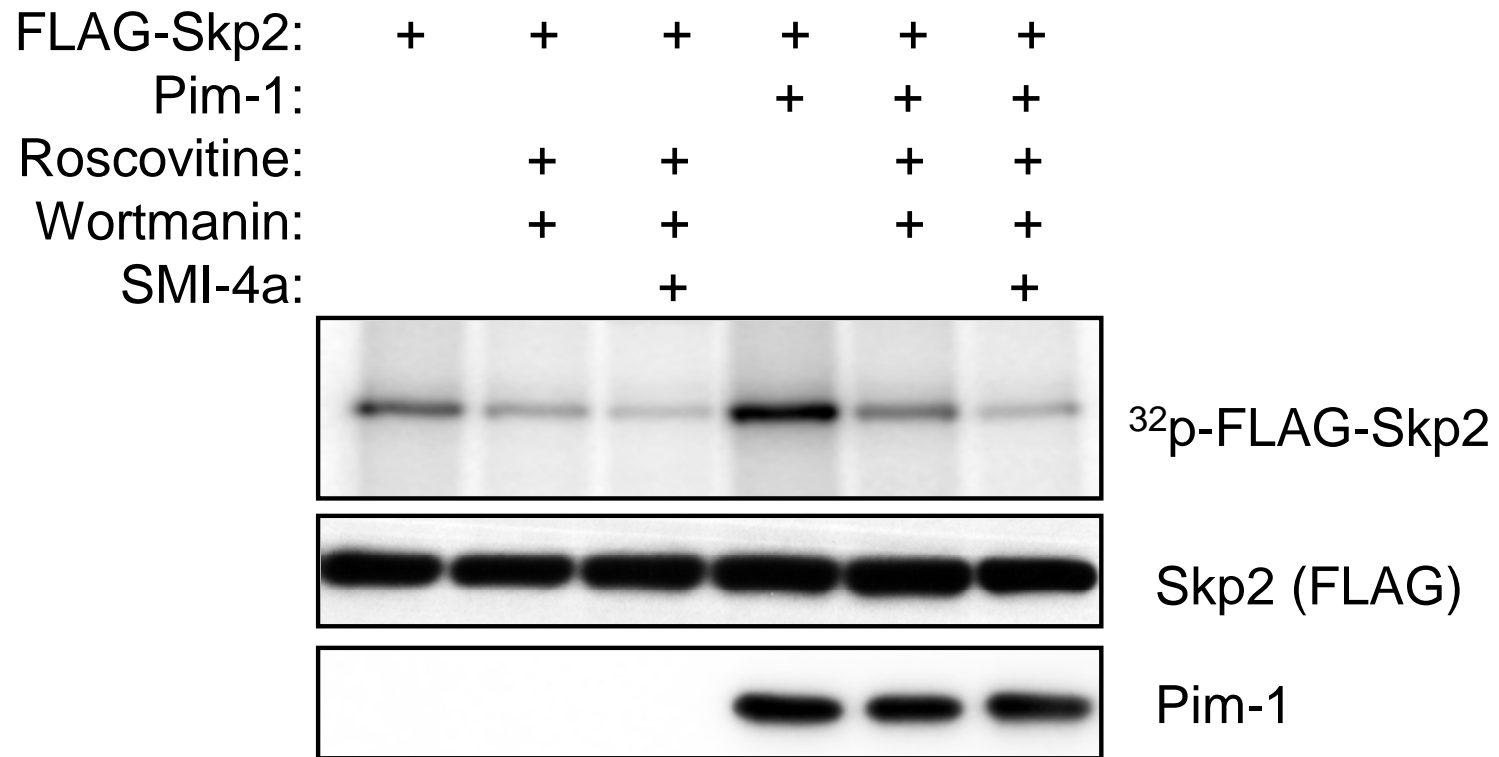
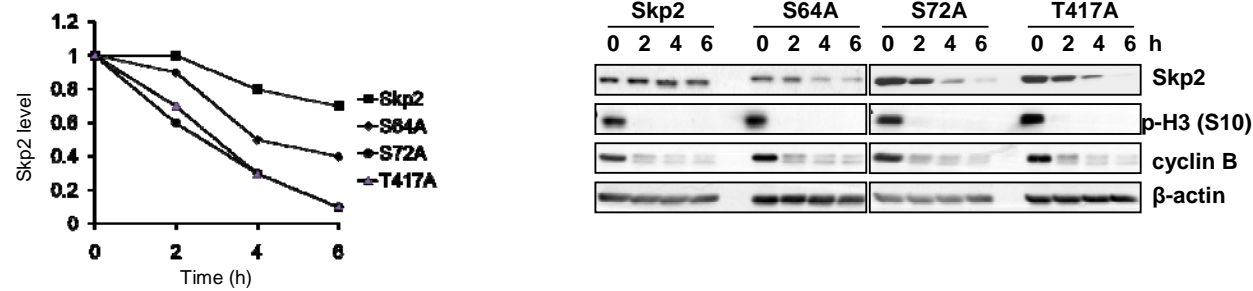


Fig. 12: HeLa cells were pretreated with roscovitrine (20 μ M), wortmannin (1 μ M), or SMI-4a (10 μ M) for 1h transfected with human Pim-1 and Skp2 and labeled with ³²P-orthophosphate followed by Flag immunoprecipitation, autoradiography (upper panel) and Flag/Pim-1 immunoblots (two lower panels).

A



B

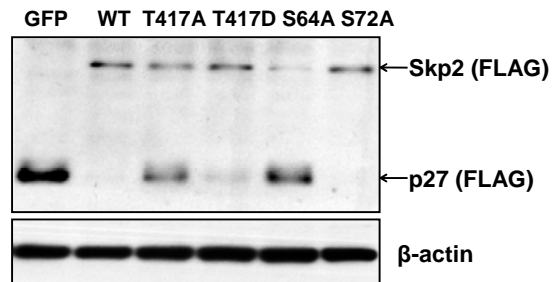


Fig. 13: (A) HeLa cells were transfected with the indicated Skp2 constructs and synchronized in M phase by mitotic shake-off of cells obtained after release from a thymidine-nocodazole block. The cells were then replated and allowed to progress through the cell cycle in the presence of cycloheximide (100 μ g/ml). Immunoblot analysis was performed at specific time points using Ab to cyclin B and phospho Histone H3 Ser10 (p-H3 (S10)) as controls. Densitometric analysis was performed using National Institutes of Health ImageJ software to quantify the expression of Skp2. Skp2 band intensity was normalized to β -actin, then normalized to the t=0 controls. (B) HEK293T cells were transfected with a FLAG-tagged p27 Skp2 constructs or a GFP

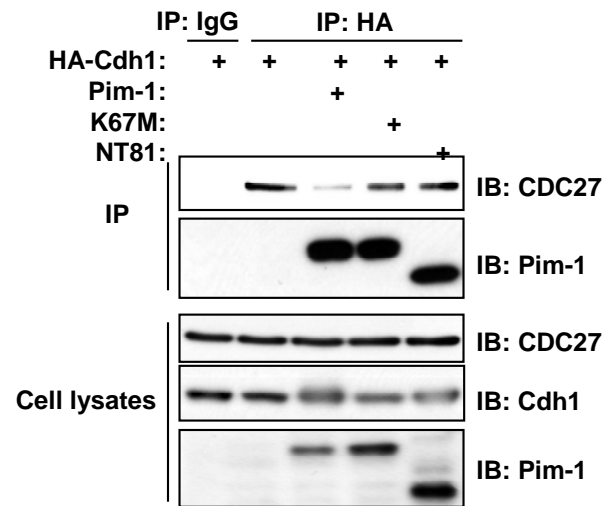
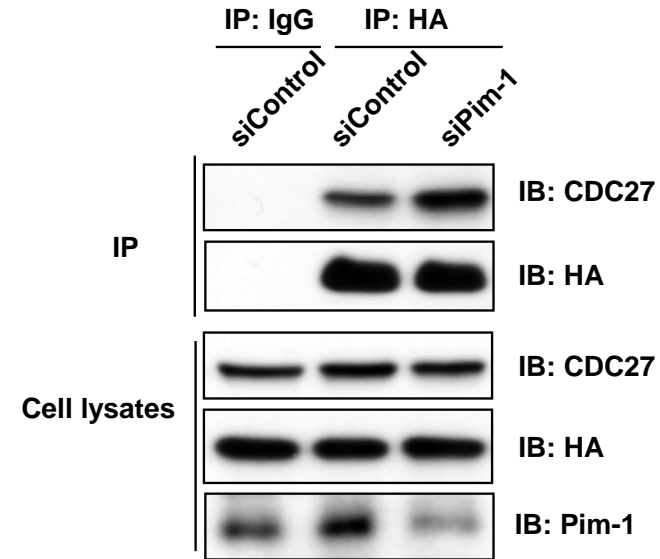
A**B**

Fig. 14: (A) HEK293T cells were transfected with HA-Cdh-1, Pim-1 or kinase dead Pim-1 K67M or NT81, immunoprecipitated with HA Ab followed by Western blotting with Abs to CDC27 and Pim-1. Lysates from these cells were immunoblotted with Ab as shown (B) HeLa cells were co-transfected with HA-Cdh1 and Pim-1 or scrambled siRNA plasmids before harvesting for co-immunoprecipitation analysis. Levels of transfected proteins in lysates were monitored by immunoblot.

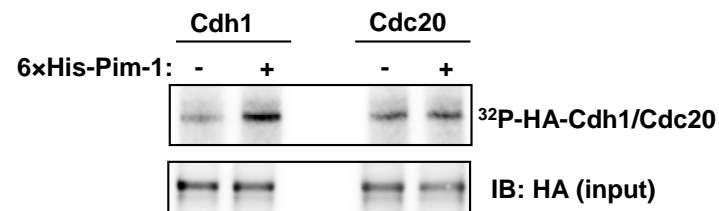
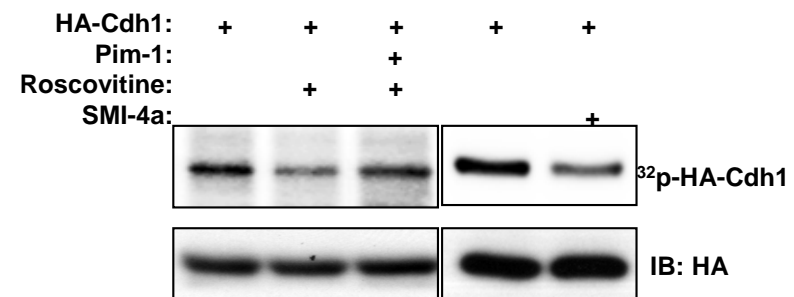
A**B**

Fig. 15: (A) In vitro translated HA-tagged Cdh1 or Cdc20 was incubated with recombinant Pim-1 and [γ - 32 P]-ATP in an in vitro kinase assay. Autoradiography (upper panel) and immunoblot (lower panel) analysis was performed. (B) HeLa cells were transfected with HA-Cdh1 and human Pim-1 and the kinase inhibitors roscovitine (20 μ M) or SMI-4a (10 μ M) were added 1h before labeling with 32 P-orthophosphate. HA-Cdh-1 was immunoprecipitated and autoradiography (upper panel).

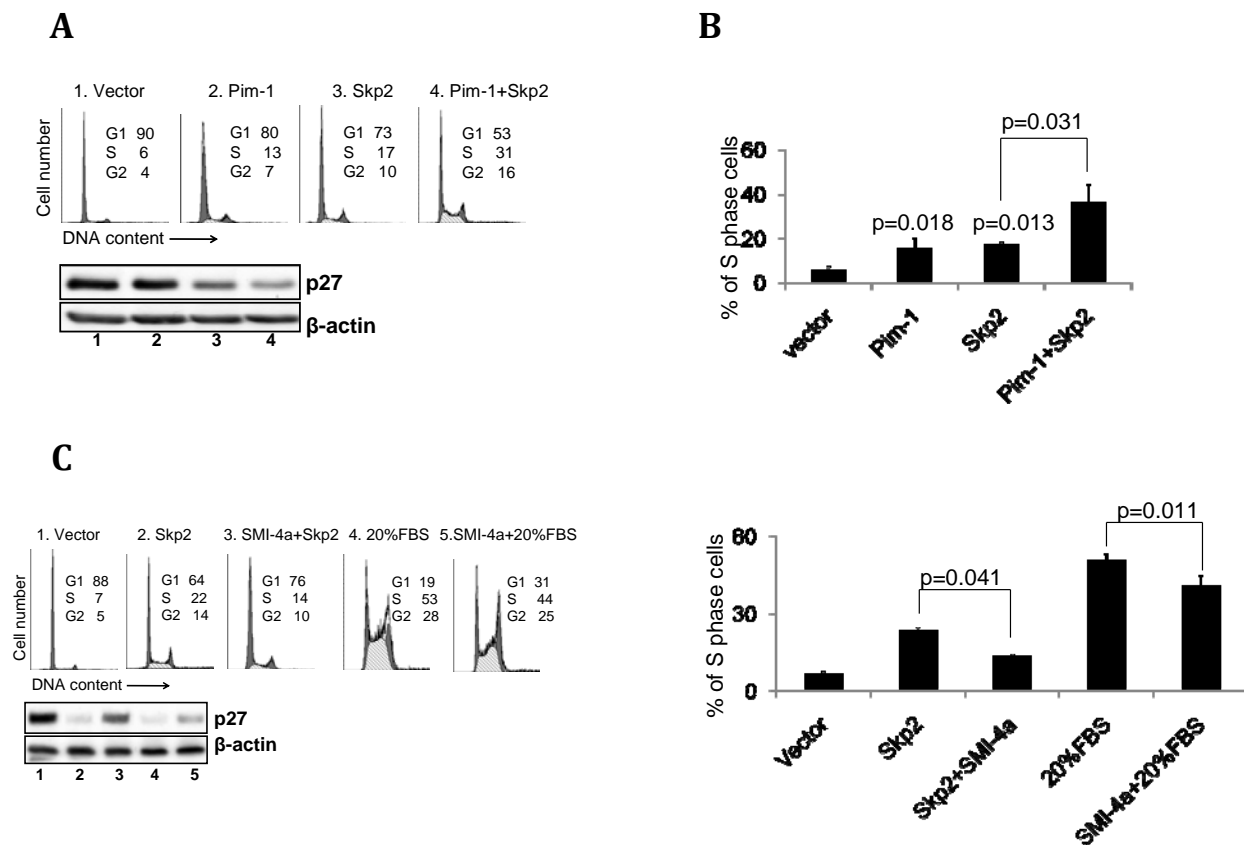


Fig. 16: (A) Rat1 cells were transduced with a lentivirus carrying indicated cDNAs. Cells were maintained in low serum conditions (0.2%) for 48 h before harvested for FACS (upper panel) and immunoblot (lower panel) analyses. (B) The percentage of S phase cells was compared to vector control, except where indicated by a bracket. (C) The experiment was performed as in (A) except that SMI-4a (5 μ M) was added 3 h before a 20% FBS stimulation (16 h). Brackets indicate a comparison of with and without SMI-4a treatment and immunoblot analysis (lower

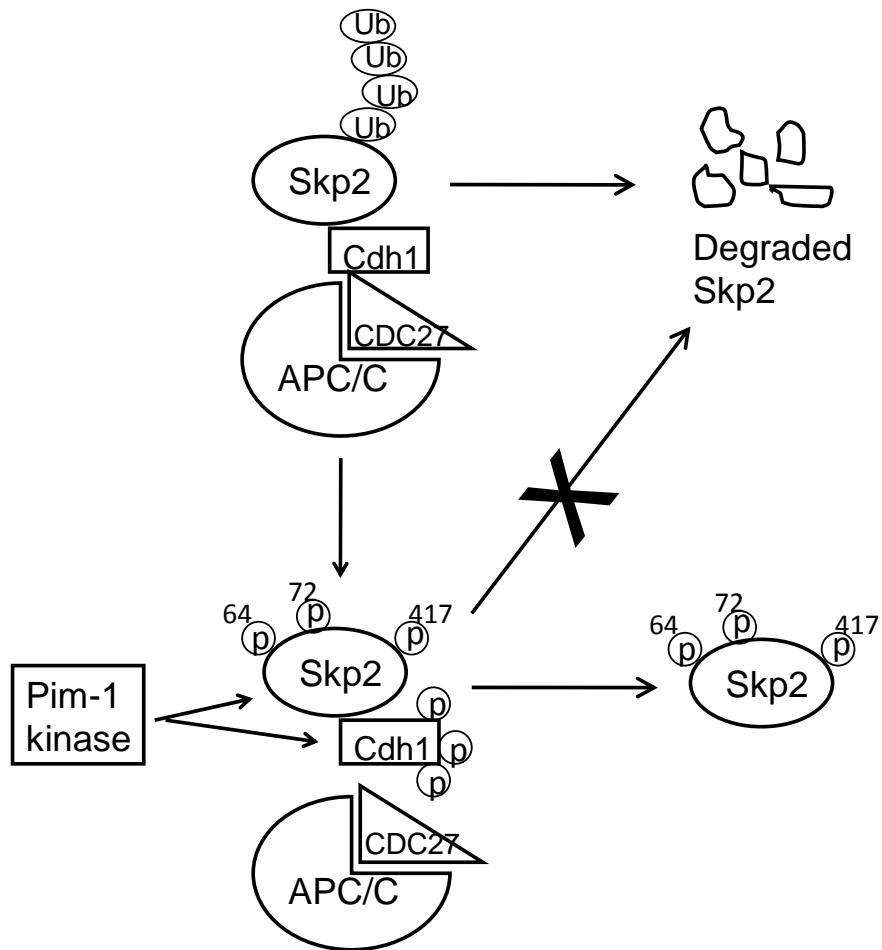


Fig. 17: A model of depicting Pim-1 regulation of Skp2 degradation. Nonphosphorylated Skp2 binds to E3 ligase APC/CCdh1 and is ubiquitinated followed by proteasome-mediated degradation. Pim-1 kinase phosphorylates both Skp2 and Cdh1 reducing Cdh1 and APC/C interaction, decreasing Skp2 ubiquitination, and consequently increasing Skp2 stability.